EXHIBIT D

Sumanirole, a Highly Dopamine D_2-Selective Receptor Agonist: In Vitro and in Vivo Pharmacological Characterization and Efficacy in Animal Models of Parkinson's Disease

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ABSTRACT

The purpose of this study is to demonstrate that sumanirole is a novel dopamine receptor agonist with high in vitro and in vivo selectivity for the D_2 receptor subtype. Sumanirole, (R)-5,6-dihydro-5-(methylamino)-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (Z)-2-butenedioate (1:1), is unique; it has greater than 200-fold selectivity for the D_2 receptor subtype versus the other dopamine receptor subtypes in radioligand binding assays. In cell-based assays, sumanirole is a fully efficacious agonist, with EC_{50} values between 17 and 75 nM. In animals, sumanirole elicits many physiological responses attributed to D_2-like receptor function. In rats, sumanirole is a full agonist for elevation of striatal acetylcholine levels (ED_{50} = 12.1 μmol/kg i.p.). Sumanirole s.c. dose dependently decreased plasma prolactin levels and depressed dopamine neuron firing rates in the substantia nigra pars compacta with an ED_{50} of 2.3 μmol/kg i.v. This high selectivity for D_2 receptors translates into excellent

locomotor stimulant activity in animal models of Parkinson's disease. In reserpinized, α-methyl-para-tyrosine-treated rats, sumanirole caused a significant and sustained increase in horizontal activity at doses $\geq$12.5 μmol/kg s.c. In unilateral 6-hydroxydopamine-lesioned rats, sumanirole caused profound, sustained rotational behavior and was substantially more efficacious than any other agonist tested. Sumanirole-stimulated rotational behavior was blocked by the dopamine receptor antagonist haloperidol. Sumanirole dose dependently improved disability scores and locomotor activities of two of three 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned monkeys. In summary, sumanirole is the first published selective D_2 receptor agonist. The compound has activity in animal models of dopamine hypofunction and has a high level of efficacy in animal models of Parkinson's disease.

Parkinson's disease results from degeneration of the dopaminergic cells in the substantia nigra and is characterized by bradykinesia, tremors, and muscular rigidity. Several dopamine receptor agonists have been used as antiparkinsonian therapy, including bromocriptine, cabergoline, pergolide, pramipexole, and ropinirole (Hagan et al., 1997). Pramipexole, cabergoline, and ropinirole are effective as monotherapy in early stage Parkinson's disease and their use significantly delays the initiation of L-dopa therapy (Inzelberg et al., 2003; Bracco et al., 2004). Dopamine agonists are also used as adjuncts to L-dopa. The combined treatment enables lower doses of L-dopa, which decreases the incidence of response fluctua-

tions and dyskinesias. However, currently available dopaminergic therapy, including agonists and L-dopa therapy, can elicit side effects, including psychiatric complications and somnolence (Lieberman, 1995). Thus, there is a need for new compounds with fewer side effects (Hobson et al., 2002; Etminan et al., 2003).

There are at least five subtypes of dopamine receptors, grouped into two subfamilies, D_1-like and D_2-like, based on pharmacological and amino acid sequence similarities (Civelli et al., 1993). The dopamine agonists bind at D_2-like dopamine receptors (Montastruc et al., 1993). The D_2-like subfamily includes D_2, D_3, and D_4 receptors. Based on mRNA distributions, it has been suggested that D_2 receptors are more abundant than in mesolimbic/mesocortical areas, whereas the D_3 and D_4 receptors are relatively more abundant in the limbic/cortical areas than in the stri-

ABBREVIATIONS: CHO, Chinese hamster ovary; MEM, minimal essential medium; HEK, human embryonic kidney; U-86170, (R)-5-(di[2,3-3H2]propylamino)-5,6-dihydro-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one; SCH23390, R-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine; SNPC, substantia nigra pars compacta; ANOVA, analysis of variance; AMPT, α-methyl-para-tyrosine; 6-OHDA, 6-hydroxydopamine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

atum (Bouthenet et al., 1991; Van Tol et al., 1991). Because of the neuroanatomical distribution of these receptors, it has been suggested that D$_3$ and D$_4$ receptors may contribute to the psychiatric disturbances that accompany dopamine agonist and L-dopa therapeutics. In fact, clozapine, a dopamine receptor blocker with high affinity for the D$_4$ subtype, has been used to ameliorate the psychoses accompanying antiparkinsonian treatment (Friedman, 1995) suggesting that this may be a D$_4$-mediated side effect. Additionally, dopamine agonists such as pramipexole and ropinirole demonstrate daytime hypersomnolence in Parkinson's disease patients, along with other sleep-related problems (Razmy et al., 2004). The receptor subtype involved in this response has not been delineated. Eliminating D$_3$- and D$_4$-activating properties of dopamine receptor agonists has the potential to reduce certain side effects of Parkinson's disease therapy.

The data in this study demonstrate sumanirole, (5R)-5,6-dihydro-5-(methylamino)-4H-imidazo[4,5,1-ij]quinolin-2(1H)-one (2Z)-2-butenedioate (1:1), is a novel dopamine agonist with high affinity and efficacy at D$_2$ dopamine receptors and has a substantial degree of selectivity for the D$_2$ receptor over other dopamine receptor subtypes. The in vitro and in vivo properties of sumanirole described here suggest that the compound may have utility in treating Parkinson's disease.

Materials and Methods

The chemical structure of sumanirole is shown in Fig. 1, and the synthesis was described previously (Heier et al., 1997). The drug has been tested as either of two salt forms: the monohydrochloride salt with a molecular weight of 239.71 g/mol and the maleate salt with a molecular weight of 319.32 g/mol.

Cell Growth. Chinese hamster ovary (CHO) cells expressing human D$_2$, D$_3$, or D$_4$ receptors or transfected with the 3C vector alone were grown in minimal essential medium [α modification (α-MEM)] supplemented with 10% fetal calf serum, 2 mM glutamine, 10 U/ml penicillin, and 100 μg/ml streptomycin. CHO-3C (mock-transfected), D2-L6, D2-S8, and D3-3 cells. HEK293 cells expressing human D$_4$ receptors were grown in Dulbecco's modified Eagle's medium high glucose supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 100 U/ml hygromycin B. D2-L6 cells express 3.5 pmol/mg human D$_{2A}$ receptors measured with [³H]spiroperidol, and 1.0 pmol/mg protein measured with [³H]U-86170, an agonist ligand. D2-S8 cells express 3.0 pmol/mg human D$_{2B}$ receptors measured with [³H]spiroperidol. D3-3 cells express D$_3$ receptors at 2.5 pmol/mg protein measured with [³H]spiroperidol (Chio et al., 1994b). HEK293 cells express D4.2 receptors at a density of 0.74 pmol/mg protein measured with [³H]spiroperidol (Chio et al., 1994a).

Receptor Binding Methods. Radioligands used were [³H]SCH23390 (D$_1$-dopamine, 70 Ci mmol, 1 nM), [³H]U-86170 (Lahti et al., 1991) (D$_2$-dopamine, 62 Ci/mmol, 2 nM), and [³H]spiperone (D$_3$ and D$_4$-dopamine, 96 Ci/mmol, 0.2 nM). Rat striatal membranes were the source of D$_1$ receptors. CHO cells and HEK293 cells expressing D$_2$, D$_3$, or D$_4$ receptors were rinsed with ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and harvested in the same

buffer. Cells were pelleted (500g, 5 min), resuspended in 25 mM Tris, 5 mM EDTA, and 5 mM EGTA, pH 7.5, and frozen in liquid nitrogen. After thawing, the cells were homogenized and centrifuged at 1000g to remove nuclei and unbroken cells. The supernatant was centrifuged at 47,000g; the membrane pellet was washed once with Tris, EGTA, EDTA, resuspended in 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl$_2$, and 1 mM EDTA, and frozen in liquid nitrogen. Membrane aliquots were stored at −70°C. For the receptor binding assays, the membranes were thawed and diluted into 20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, or 1 mM EDTA, 10 mM MgSO$_4$. Nonspecific binding was determined using 3 μM SCH23390 (D$_1$ receptor antagonist) or 3 μM haloperidol (D$_2$, D$_3$, and D$_4$ receptor antagonist). Binding reactions were carried out in 0.9-ml total volume for 1 h at room temperature. Reactions were stopped by vacuum filtration. Competition binding experiments employed 11 concentrations of test compound run in duplicate. IC$_{50}$ values were determined by fitting the data to a one-site model by nonlinear least-squares minimization. K_i values were calculated with the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

cAMP Measurements. cAMP accumulation was measured in intact CHO cells plated at a density of 15,000 cells/well in a 24-well plate 48 h before the experiment. The cells were incubated in serum-free medium 1 h before the experiment. Fresh medium (0.5 ml) containing 100 μM forskolin, 100 μM isobutyl methylxanthine, and varying concentrations of drugs were added to each well, and cAMP was allowed to accumulate for 15 min at room temperature. The reactions were terminated by the removal of the medium and the addition of 100 μl of cold 7.5% trichloroacetic acid. The samples were diluted by the addition of 1.0 ml of 50 mM sodium acetate, pH 6.2, and aliquots were assayed by radioimmunoassay using the Biomedical Technologies Incorporated (Stoughton, MA) cAMP radioimmunoassay kit.

Mitogenesis Assays. CHO cells were seeded into 96-well plates at a density of 5000 cells/well and were grown at 37°C in a α-MEM, 10% fetal calf serum for 48 h. The wells were rinsed three times with serum-free α-MEM. Ninety microliters of fresh α-MEM was added along with 10 μl of drug (diluted in sterile water and filtered through 0.2-μm filters) or sterile water alone. Eight wells of cells received 100 μl of α-MEM with 10% fetal calf serum. After culture for 16 to 17 h, [³H]thymidine (1 μCi/well) was added for 2 h. The cells were trypsinized and harvested onto filter mats with a Skatron cell harvester (Molecular Devices, Sunnyvale, CA). The filters were counted in a Betaplate counter (PerkinElmer Life and Analytical Sciences, Boston, MA).

Measurement of [³H]Arachidonic Acid Release. Cells were plated in 24-well plates at a density of 15,000 cells/well 48 h before use. Cells were labeled by incubation with [³H]arachidonic acid (210 Ci/mmol, 0.4 μCi/ml; Amersham Biosciences Inc., Piscataway, NJ) in α-MEM (1 ml) supplemented with 10 mM HEPES, pH 7.5, and 0.5% fatty acid-free bovine serum albumin for 3 h at 37°C. The cells were then washed twice with 1 ml of the same buffer. Drugs were added in 1 ml of the same buffer, and the cells were incubated at 37°C for 30 min. Samples (0.5 ml) from each well were counted by liquid scintillation spectroscopy.

In Vivo General Methods, Rat. Unless otherwise stated, the following generalizations apply to all in vivo rat testing. Animals were male Sprague-Dawley rats from Harlan (Indianapolis, IN) and were maintained in a temperature-controlled environment at 22 ± 1°C, with a 12:12-h light/dark cycle (lights on from 6:00 AM to 6:00 PM). They were group-housed and received food (Rodent Laboratory Chow #5001; Purina Mills Inc., Richmond, IN) and water ad libitum. All rodent testing was in compliance with the Animal Welfare Act Regulations, 9CFR Parts 1, 2, and 3, and with the Guide for the Care and Use of Laboratory Animals (National Academy Press, National Academy of Sciences, 1996).

Electrophysiology. Rats weighing between 280 and 330 g were anesthetized with chloral hydrate (400 mg/kg i.p.). The femoral artery and vein were catheterized for monitoring blood pressure and
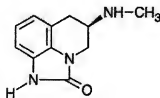

Fig. 1. Chemical structure of sumanirole.

administration of drugs, respectively. Glass microelectrodes filled with pontamine sky blue in 2 M NaCl used for extracellular recordings were lowered through a small hole burred through the calvarium by means of a hydraulic microdrive. Stereotaxic coordinates for placement of recording electrodes were $AP_r -4.8$ to -5.0; $L_r +2.0$; and $V_r -6.8$ to -7.8 mm, relative to bregma (Paxinos and Watson, 1986). Substantia nigra pars compacta (SNPC) neurons were identified by waveform and firing patterns (Bunney et al., 1973). Histological localization of iontophoresed pontamine sky blue spots verified electrode locations. Drug solutions were made in distilled water with equimolar citric acid added as needed. Drug effects were measured as changes in firing rates monitored by an integrated rate meter.

Plasma Prolactin. Male Long Evans rats weighing 200 to 225 g (Harlan) were used in this assay. Sumanirole was dissolved in saline and administered s.c. in a volume of 1 ml/kg at various times. Thirty minutes after drug or vehicle administration, animals were killed by decapitation, and trunk blood was collected and plasma was stored. Plasma prolactin was measured by a double-antibody radioimmunoassay using the reagents and procedures of the National Institute of Diabetes and Digestive and Kidney Diseases assay kit (kindly supplied by Drs. A. F. Parlow and S. Raiti, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program, Torrance, CA). National Institute of Diabetes and Digestive and Kidney Diseases rat prolactin (RP-3) was used as the standard. Using a 100-μl aliquot of plasma, the lower limit of sensitivity for prolactin was 0.1 ng/ml. The intra-assay coefficient of variation is usually about 8.6%.

Striatal Acetylcholine Concentration. Rats (130–150 g; Charles River Laboratories, Inc., Wilmington, MA) were used in this study. Solutions of sumanirole and ropinirole were prepared in 0.25% carboxymethylcellulose. Drug treatments were given i.p., and control rats received an equal volume of vehicle (2 ml/kg). Thirty minutes after treatment, animals were killed by decapitation; the brain was quickly removed from the skull and placed in ice-cold 0.32 M sucrose. Bilateral striata were dissected and homogenized in 0.05 N perchloric acid containing ethylhomocholine as an internal standard. Acetylcholine concentration was determined by high-pressure liquid chromatography. Each point represents five to six animals, and statistical analysis was done by a one-way ANOVA, followed by Student's t test.

Locomotor Activity Measurements in Reserpinized Rats. Rats pretreated with reserpine and the dopamine synthesis inhibitor α-methyl-*para*-tyrosine (AMPT) are akinetic and cataleptic. These effects can be reversed by L-dopa (Carlsson et al., 1957). Reserpine/AMPT-treated rats have been used as a model of dopamine depletion to mimic parkinsonian conditions. Rats weighing 200 to 250 g were used in this assay. Reserpine and haloperidol were purchased from Sigma/RBI (Natick, MA). Sumanirole was dissolved in physiological saline (0.9% NaCl), whereas haloperidol was dissolved in a few drops of glacial acetic acid and further diluted with 5.5% glucose solution. All compounds were administered s.c. in a volume of 5 ml/kg. Animals were pretreated with reserpine (5 mg/kg s.c., 18 h prior) and AMPT (100 mg/kg s.c., 1 h prior) before the experiment. The animals were injected with sumanirole or saline, and locomotor activity was measured using animal activity monitors (Digiscan model RXYZM TAO; Omnitech Inc., Columbus, OH). Data are presented as horizontal counts expressed as percentage of saline-treated controls (means ± S.E.M.) and comparisons were done at discrete 10-min intervals, comparing vehicle to individual doses of drug. Statistical analysis was done by one-way ANOVA, followed by an unpaired t test; on a point-by-point basis, any data differing from vehicle at $p \leq$ 0.05 were considered an increase in activity.

Turning in 6-Hydroxydopamine (6-OHDA)-Lesioned Rats. Unilateral 6-OHDA injections into the substantia nigra cause selective destruction of dopamine neurons, leading to supersensitivity of the dopamine receptors in the caudate putamen on the injected side. In these animals, dopamine receptor agonists cause contralateral turning (Ungerstedt, 1971).

Rats (225–280 g) were pretreated with desmethylimipramine 25 mg/kg i.p. 1 h before surgery. They were anesthetized with Chloropent (Sigma Chemical Co., St. Louis, MO) given at 3 ml/kg i.p. and placed in a stereotaxic apparatus with the incisor bar raised to 4 mm (smaller rats) or 5 mm (larger rats). A small hole was drilled through the skull and a 30-gauge stainless tubing was lowered to the right substantia nigra using the following coordinates: for smaller rats, AP, −1.5 mm; L, +1.8 mm; and V, −8.0 mm; for larger rats, AP, −2.8 mm; L, +2.0 mm; and V, −8.0 mm (Pellegrino et al., 1979). 6-OHDA, hydrogen bromide solution was injected into the substantia nigra at 12 μg/2 μl free base) in 0.9% saline/0.1% ascorbic acid at 1 μ/min, using a Hamilton syringe pump (Hamilton Co., Reno, NV). After surgery, the scalp incision was closed with stainless steel wound clips, and Mycitracin Plus (Johnson & Johnson, Skillman, NJ) ointment containing lidocaine and antibiotics (bacitracin, neomycin, and polymyxin B) was applied to the incision area. Rats were replaced in group housing, and clips were removed after 1 week.

Two weeks after surgery, the efficacy of the lesions were tested by monitoring the turning rate of rats given 0.5 mg/kg s.c. apomorphine HCl in 0.9% saline/0.1% ascorbic acid. Total turns were recorded at 10-min intervals in automated monitors (Rotoscan; Omnitech Inc.). Each rat was connected by a lightweight harness and tether to a rotometer at the top of a clear plastic cylindrical cage (11 in. in diameter). The cage floor paper was changed for each animal. Rats were used for sumanirole experiments if they had at least 30 turns/10 min (range 30–160 turns/10 min) in this screen. Whenever possible, animals were balanced across treatment groups to accord each treatment a similar mix of animals from within this range. Groups were compared statistically by an F-test for variance of means, followed by a one-way analysis of variance and Student's t test for individual data points and area under the curve. Analysis was done using RS/1 software (Brooks Automation Inc., Chelmsford, MA), conducted in-house.

MPTP-Lesioned Monkeys. Three female cynomolgus monkeys (*Macaca fascicularis*), weighing approximately 3 kg each, were treated with MPTP i.v. at different dosages until variable, but stable, parkinsonian features occurred. The monkeys were rated previously across a number of dopamine agonists. Sumanirole was injected s.c. During the period of peak drug effect, behavioral responses were scored every 30 min using an MPTP monkey disability scale (Gomez-Mancilla et al., 1993), and locomotor activity was recorded every 15 min by photocells mounted in the cages. Results obtained with L-dopa/benserazide 50:12.5 mg/kg (Prolopa; Roche Diagnostics, Mississauga, ON, Canada) and 0.1 mg/kg apomorphine were used as reference standards.

Results

Studies with Cloned Receptors. Sumanirole is an agonist selective for the D_2 subtype of dopamine receptors (Table 1). To measure high-affinity, guanine nucleotide-sensitive agonist interactions at D_2 receptors, an agonist ligand, [³H]U-86170, was used (Lahti et al., 1991). The affinity of sumanirole for D_2 receptors is 9.0 ± 1.0 nM. This is similar to the affinity of D_2 receptors for ropinirole and slightly higher

TABLE 1
Binding affinities of agents at dopamine receptors
Values are K_i ± S.E.M. ($n = 3$–6).

Receptor	Binding Affinities of Drugs at Dopamine Receptors			
	Sumanirole	Ropinirole	Bromocriptine	Pergolide
	nM			
D_2	9.0 ± 1	7.2 ± 0.9	27 ± 9	1 ± 0.2
D_3	1940 ± 142	22 ± 4	18 ± 2	0.4 ± 0.03
D_4	>2190	1450 ± 390	373 ± 15	9.3 ± 1
D_1	>7140	>7140	3420 ± 130	1300 ± 132

and lower than the receptor affinities for bromocriptine and pergolide, respectively, all agents used clinically for the treatment of Parkinson's disease (Eden et al., 1991; Keyser et al., 1995; Rascol et al., 1996). The affinity of D$_3$, D$_4$, and D$_1$ receptors for sumanirole was calculated to be at least 200-fold lower than at D$_2$ receptors. In contrast, ropinirole, bromocriptine, and pergolide all showed high affinity for D$_3$ receptors; pergolide also showed high affinity for the D$_4$ receptor subtype.

We next examined whether sumanirole is an agonist or antagonist at the D$_2$ receptor using three measures: cAMP, arachidonic acid release, and mitosis. Forskolin-stimulated cAMP accumulation in CHO cells (Fig. 2). Both compounds caused near maximal inhibition of forskolin-stimulated cAMP at the highest concentrations. Sumanirole had no effect on forskolin-stimulated cAMP levels in CHO-3C cells (transfected with vector alone) at concentrations up to 1 μM, suggesting that the response in L6 cells occurs via the transfected D$_2$ receptor. Table 2 compares the potencies of sumanirole, ropinirole, and dopamine in two assays for activation of D$_2$ receptors in CHO-L6 cells: cAMP inhibition and potentiation of ATP-stimulated arachidonic acid release (Lajiness et al., 1994). Sumanirole is approximately 3 times less potent than ropinirole at each measurement (Table 2). Both compounds were as efficacious as dopamine at maximal concentrations, indicative of high levels of intrinsic activity. Sumanirole activated D$_2$ receptor-stimulated mitogenesis with an EC$_{50}$ of 4.6 nM, but it had no activity at D$_3$ and D$_4$ receptors in measurements of receptor-stimulated mitogenesis at concentrations up to 1 μM; this was consistent with the very low affinity of these receptors for this compound. In comparison, ropinirole potentiates activated D$_2$ and D$_3$ receptors in the mitogenesis assay [EC$_{50}$ values of 12 $\pm$ 1.0 nM (n = 5) and 7.5 $\pm$ 2.9 nM (n = 5), respectively].

Studies in Animals. Dopamine agonists that activate the D$_2$-like subfamily of dopamine receptors inhibit the release of prolactin from anterior pituitary cells (Ho and Thorner, 1988). The effects of four doses of sumanirole on plasma prolactin levels in male Sprague-Dawley were tested. Sumanirole decreased plasma prolactin at all doses studied with

TABLE 2
In vitro D$_2$ potency of sumanirole and other agonists
Data are mean EC$_{50}$ $\pm$ S.E.M. for n shown in parentheses.

Assay	Potency Measurements at D$_2$ Receptors		
	Sumanirole	Ropinirole	Dopamine
		nM	
cAMP Inhibition	17 $\pm$ 4.8 (4)	5.6 $\pm$ 0.6 (3)	13[a]
[^{3}H]Arachidonate release	75 $\pm$ 16 (6)	19 $\pm$ 4.1 (3)	31 $\pm$ 9.3 (5)
Mitogenesis	32 $\pm$ 6.4 (3)	12 $\pm$ 1.0 (5)	5.4[a]

[a] With permission from Lajiness et al. (1994).

significant decreases at all doses (Table 3). The effects at 31 μmol/kg lasted at least 120 min postinjection.

Dopamine agonists depress dopamine neuron firing rates in the SNPC by activation of presynaptic dopamine receptors of the D$_2$-like subfamily. As demonstrated by population dose-response curves, sumanirole resulted in inhibition of SNPC dopamine neuronal firing with an ED$_{50}$ $\pm$ S.E.M. of 2.3 $\pm$ 0.9 μmol/kg i.v. (n = 6) (Fig. 3). Complete inhibition of basal firing rate was seen at 16.8 μmol/kg i.v. The inhibition of cell firing was reversed by haloperidol.

Activation of striatal postsynaptic D$_2$-like receptors decreases acetylcholine release and raises striatal acetylcholine concentrations (Sethy, 1979). Administration of both ropinirole and sumanirole to rats caused a dose-dependent increase in striatal acetylcholine levels (Fig. 4). The maximal effects of both compounds were similar, with a near doubling of striatal acetylcholine concentration. The ED$_{50}$ values for the effects (calculated as the fitted half-maximal response $\pm$ S.E.M. of the fit using the dose-response fit equation of Sigma Plot (SPSS Inc., Chicago, IL) were 6.0 $\pm$ 8.2 μmol/kg i.p. for ropinirole and 12.1 $\pm$ 4.1 μmol/kg i.p. for sumanirole.

Effects in Animal Models of Parkinson's Disease. Sumanirole increased locomotor activity in reserpine/AMPT-pretreated rats with pronounced effects at 12.5 μmol/kg s.c. (Fig. 5). The onset of this effect was approximately 30 min postinjection, and the duration of the 12.5 μmol/kg dose was at least 2.5 h. Even greater locomotor activation was observed in reserpinized/AMPT-treated rats at 42 μmol/kg s.c., and the activation was completely blocked by 0.3 mg/kg hal-
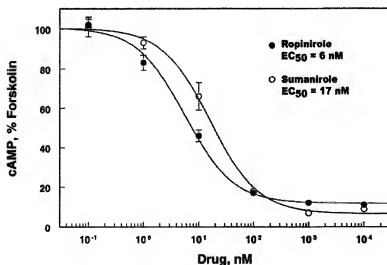

Fig. 2. Dose-dependent inhibition of forskolin-stimulated cAMP in CHO cells expressing recombinant D$_{2A}$ receptors by sumanirole and ropinirole. Each point is a mean $\pm$ S.E.M. of triplicate determinations expressed as percentage of the 100 μM forskolin response. Concentration of drug given as nanomolar. $\bullet$, ropinirole; $\bigcirc$, sumanirole.

TABLE 3

Sumanirole versus plasma prolactin levels in male rats

Data are mean ± S.E.M. for n data in parentheses. Statistical analysis was carried out using ANOVA.

Drug Dose (30 min)	Prolactin
	ng/ml
Vehicle	3.93 ± 0.88 (6)
Sumanirole, 3.1 μmol/kg	0.77 ± 0.10*(6)
Sumanirole, 9.4 μmol/kg	n.d. (8)*
Sumanirole, 31 μmol/kg	n.d. (8)*
Sumanirole, 94 μmol/kg	0.65 ± 0.03*(5)

n.d., below detection limits.
* Significant decrease at $p = 0.05$.

operidol. These data suggest that sumanirole induces strong behavioral activation through a D_2 dopamine receptor.

Sumanirole was also highly efficacious at causing contralateral turning in unilateral 6-OHDA-lesioned rats after either oral or s.c. administration. A dose-response and time-course study of sumanirole given orally at 4.2, 12.5, or 42 μmol/kg showed turning activity in substantia nigra-lesioned rats, with significant effects seen at the two higher doses (Fig. 6). Maximal turning behavior was reached at 2 to 3 h after oral administration. Additionally, in this assay, suma-

nirole had a long duration of action as 12.5 and 42 μmol/kg still showed near-maximal efficacy at 4.5 h postinjection.

Ropinirole also caused dose-dependent increases in contralateral turning in the 6-OHDA-lesioned rats (Fig. 7). Significant turning behavior was measured at 19 μmol/kg s.c. A maximally effective dose of ropinirole was not as efficacious as sumanirole, as measured by the degree of turning. In the same set of rats, sumanirole (12.5 μmol/kg s.c.) induced approximately 3 times as many turns per 10-min interval than 19 μmol/kg s.c. ropinirole. Note that the effects of 12.5 μmol/kg sumanirole s.c. (Fig. 6) are equivalent to the effects produced by 42 mol/kg sumanirole administered orally (Fig. 7). Thus, the oral potency is approximately one-third the potency of the parenteral route. The peak response of sumanirole was also greater than the maximally effective doses of the following drugs given s.c.: 0.37 μmol/kg apomorphine, 4.6 μmol/kg bromocriptine, 9.5 μmol/kg pergolide, 13 μmol/kg quinpirole, and 11.7 μmol/kg SKF 38393. The turning activity induced by sumanirole is completely blocked by the coadministration of 0.27 or 2.7 μmol/kg haloperidol (Fig. 8), indicating that turning is mediated by a D_2-like dopamine receptor.

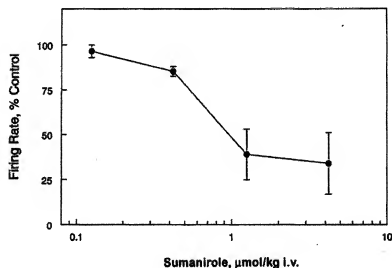Drug-experienced cynomolgus monkeys rendered parkin-

Fig. 3. Dose-dependent inhibition of the firing rate of dopamine cells in the substantia nigra pars compacta by sumanirole. The population dose-response curve for depression of rat SNPC dopamine neurons ($n = 8$) by sumanirole i.v. is shown. The ordinate is the firing rate relative to predrug control (mean ± S.E.M.); the abscissa is the cumulative i.v. doses in micromoles per kilogram.
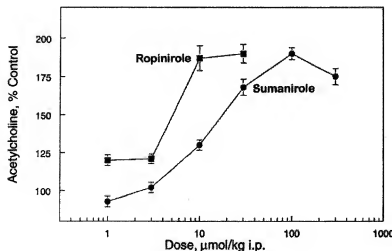

Fig. 4. Dose-dependent increases in striatal acetylcholine concentration by ropinirole and sumanirole. Striatal acetylcholine was measured in rats 30 min after i.p. injections of ropinirole or sumanirole, with doses listed in micromoles per kilogram. ■, ropinirole; ●, sumanirole.
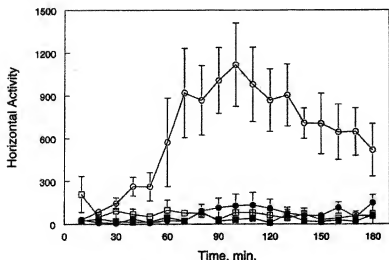
Fig. 5. Increases in locomotor activity in reserpinized rats treated with sumanirole s.c. Horizontal locomotor activity was measured as described under *Materials and Methods*; rats were pretreated with reserpine (5 mg/kg s.c., 18 h) and α-methyl-*para*-tyrosine (100 mg/kg s.c., 1 h). Sumanirole or vehicle was injected s.c. at time 0, and locomotor activity was recorded at discrete 10-min intervals. ●, saline, 5 ml/kg, n = 4. Sumanirole doses: ■, 1.25 μmol/kg, n = 4; □, 4.2 μmol/kg, n = 4; ○, 12.5 μmol/kg, n = 4.
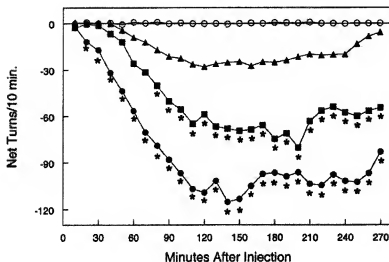

Fig. 6. Effects of oral sumanirole on turning behavior in rats with unilateral 6-OHDA lesions of the substantia nigra. Turning behavior of rats with unilateral 6-OHDA lesions of the substantia nigra was measured after injections of vehicle (○), n = 5; 4.2 μmol/kg sumanirole (▲), n = 4; 12.5 μmol/kg sumanirole (■), n = 7; and 42 μmol/kg sumanirole (●), n = 6. *, p < 0.05 significance compared with controls by F-test for variance of means followed by one-way ANOVA with t test for individual points.

sonian with MPTP-induced lesions of the substantia nigra were treated with different dosages of sumanirole and tested for improvement in scores using a MPTP monkey disability scale (Fig. 9). Two monkeys (A and B) had dose-dependent reductions in the disability scores after sumanirole, especially at the 12.5 μmol/kg dose. Monkey A had a dramatic recovery at 2.5 μmol/kg s.c. lasting for 5 to 6 h after a delay of 0.5 to 1 h. This monkey also had a dyskinesia accompanying the recovery. Monkey B required 12.5 μmol/kg sumanirole to show a dramatic response, and the response also lasted for 5 to 6 h after a 0.5 to 1 h delay. No dyskinesias were observed in monkey B to either sumanirole or other dopamine agonists. Monkey C did not show dramatic improvement even with the 12.5 μmol/kg dose; however, this monkey was unresponsive to apomorphine as well. In locomotion measurements, sumanirole at 12.5 μmol/kg s.c. increased the activity of monkeys A and B more than Prolopa. Monkey C did not increase locomotion with sumanirole or with apomorphine, although this monkey did respond to Prolopa (Fig. 10).

Discussion

The striking difference between sumanirole and the other examined dopamine agonists (ropinirole, bromocriptine, and pergolide) lies in sumanirole's lack of affinity for D₃ and D₄ receptors, whereas the other dopamine agonists all showed high affinity for D₃ and/or D₄ receptors. Sumanirole is an imidazoquinolinone and the first compound described as a highly selective agonist for D₂ receptors over the other D₂-like subtypes, D₃ and D₄. The selectivity of this compound was demonstrated in radioligand binding assays, where it has at least 200-fold higher affinity for D₂ receptors than for the other dopamine receptor subtypes. The other dopamine agonists examined did not demonstrate D₂ receptor-selectivity. Sumanirole has been shown to be inactive in more than 80 enzyme and receptor assays. The compound does have moderate affinity at the 5-HT$_{1A}$ receptor (K_i = 95 nM; unpublished observations).

D₂ receptors are abundant in motor areas such as the basal ganglia; therefore, their activation may be associated with therapeutic efficacy of dopamine agonists in Parkinson's disease. In contrast, D₃ and D₄ receptors are located in limbic and cortical areas and may contribute to the psychiatric disturbances that accompany dopamine agonist and L-dopa therapeutics. In support of this hypothesis, clozapine, a dopamine receptor blocker with higher affinity for the D₄ subtype, has been used to ameliorate the psychoses accompany-
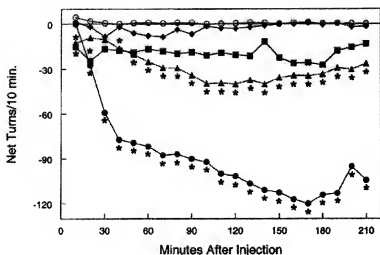
Fig. 7. Comparison of dose-response ropinirole s.c. to a single dose of sumanirole s.c. on turning behavior in unilaterally 6-OHDA-lesioned rats. Turning behavior of rats with unilateral 6-OHDA lesions of the substantia nigra was measured after injections of vehicle (O), $n = 13$; 0.38 μmol/kg ropinirole s.c. (◆), $n = 4$; 3.8 μmol/kg ropinirole s.c. (■), $n = 6$; 19 μmol/kg ropinirole s.c. (▲), $n = 8$; and 12.5 μmol/kg sumanirole s.c. (●), $n = 6$. *, $p < 0.05$ significance compared with controls after F-test for variance of means, followed by one-way ANOVA with t test for individual points.
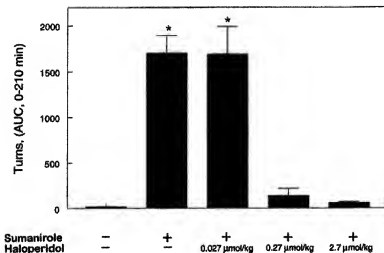

Fig. 8. Haloperidol antagonism of sumanirole-induced turning behavior in unilaterally 6-OHDA-lesioned rats. Turning behavior of rats with unilateral 6-OHDA lesions of the substantia nigra was measured after injections of either vehicle or 12.5 μmol/kg sumanirole and the indicated concentrations of haloperidol. Each bar is the total area under the curve ± S.D. ($n = 4$) for turning behavior from 0 to 210 min postinjection. *, $p < 0.05$ significance when compared with controls after F-test for variance of means, followed by one-way ANOVA with t test for area under curve.

ing antiparkinsonian treatment (Friedman, 1995). D_3 receptor agonists have also been shown to decrease locomotor activity (Lagos et al., 1998). This suggests that sumanirole's unique selectivity may confer beneficial effects in the treatment of Parkinson's disease. Eliminating D_3- and D_4-activating properties of dopamine receptor agonists may potentially reduce nonmotor side effects and improve tolerability. Whether the unique selectivity of sumanirole translates into a clinical advantage over other dopamine agonists remains to be determined.

The unique selectivity of sumanirole allows the compound to be used to characterize D_2-specific activity in the central nervous system that had previously been only inferred by using nonselective D_2-like receptor agonists. Sumanirole inhibits prolactin release. D_2 receptor mRNA is found in the pituitary gland (Bunzow et al., 1988; Sokoloff et al., 1990), and D_2 receptors inhibit prolactin release from lactotrophs (Missale et al., 1991). Thus, it is not surprising that the D_2-selective agonist sumanirole can mediate the prolactin inhibition response, but these results provide evidence that sumanirole has anticipated in vivo D_2 activity. D_2-like receptors inhibit the firing rates of SNPC dopamine neurons (Pinnock, 1984; Piercey et al., 1996a,b).

Both mRNA for D_2 and D_3 receptors have been localized to these dopaminergic neurons (Meador-Woodruff et al., 1989; Bouthenet et al., 1991). Sumanirole inhibited the firing rate of these cells in a similar dose range as the doses shown to inhibit prolactin release. These findings support a role for the molecularly defined D_2 receptors as autoreceptors that regulate the firing rates of SNPC neurons. Furthermore, activation of D_2-like receptors decreases acetylcholine release (Consolo et al., 1987). Changes in acetylcholine content in the striatum were used as an in vivo measure of D_2-like receptor activation. By this measurement, sumanirole is equally efficacious in vivo as other compounds used as antiparkinsonian agents. In three animal models of Parkinson's disease, sumanirole was shown to have good locomotor stimulant properties, including reserpine/AMPT-treated rats, 6-OHDA-lesioned rats, and MPTP monkeys. Reserpine treatment of rats to deplete catecholamine stores results in a hypokinetic animal that can be activated by L-dopa and provides an animal model for Parkinson's disease (Carlsson et al., 1957). Reserpine-induced akinesia was dose dependently reversed by sumanirole and the activation effect was blocked by haloperidol. The mechanism for this locomotor activation is likely to be direct stimulation of postsynaptic D_2 dopamine receptors.
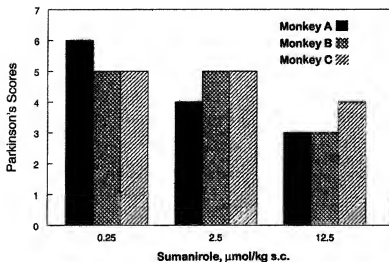
Fig. 9. Dose-dependent changes in MPTP-induced disability symptoms in cynomolgus monkeys by sumanirole. Using a disability scale for MPTP monkeys (Gomez-Mancilla et al., 1993), three monkeys were evaluated for changes in disability after s.c. injection of sumanirole with doses listed in micromoles per kilogram. During the period of peak drug effect, the behavioral responses were scored every 30 min. Each bar represents the score from monkey A, B, or C at each dose, respectively.
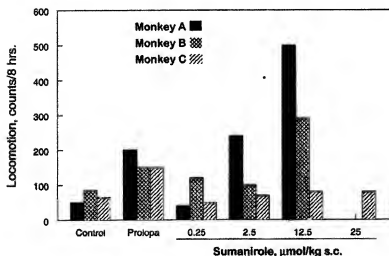

Fig. 10. Dose-dependent increases in locomotor activity in MPTP-lesioned cynomolgus monkeys by sumanirole; comparisons with Prolopa. Changes in locomotor activity by sumanirole and Prolopa were evaluated in the same three monkeys shown in Fig. 9 via recordings made every 15 min by photocells mounted in the cages. Each bar represents the total locomotor activity counts per 8 h for each individual monkey.

Sumanirole was highly efficacious at causing contralateral turning in unilateral 6-OHDA-lesioned rates after either oral or s.c. administration, demonstrating superior efficacy over other dopamine agonists tested in this model. Ropinirole was slightly more potent but equally efficacious as sumanirole in assays of D$_2$ receptor activation in cell-based assays, and at D$_2$-like receptor activation in in vivo responses. However, even maximally effective doses of ropinirole did not produce the same degree of response as sumanirole on rotational behavior. Furthermore, sumanirole was more efficacious than other agonists (bromocriptine, pergolide, and apomorphine) tested at maximally effective doses in this model. The enhanced efficacy of sumanirole in the 6-OHDA-lesioned rat model could be a result of other undiscovered activities of this compound; however, turning was completely blocked by the dopamine receptor antagonist haloperidol. This suggests the enhanced efficacy in this model resulted from sumanirole's unique D$_2$-receptor selectivity.

MPTP-lesioned monkeys represent another animal model for Parkinson's disease. The toxin MPTP causes selective destruction of dopaminergic neurons resulting in behavioral deficits similar to those observed in diseased humans (Burns et al., 1983). L-Dopa and dopamine agonists reverse the behavioral deficits in MPTP-treated monkeys. Chronic L-dopa can also cause dyskinesias in monkeys as it does in patients with Parkinson's disease (Bedard et al., 1986). Sumanirole has only been tested to a limited extent in the MPTP-lesioned monkey model and only in monkeys tested extensively with other dopaminergic agents. Sumanirole caused improvement in Parkinson's disability scores in two of the three monkeys. Sumanirole also increased the locomotion of the two responding monkeys to a greater extent than Prolopa. This is consistent with the high efficacy effects of sumanirole in the rat 6-OHDA turning model. The studies in the MPTP-lesioned monkey suggest that sumanirole is efficacious in this model but are too limited to draw solid conclusions about the relative efficacy or the dyskinetic potential of this compound. The current issue of *Journal of Pharmacology and Experimental Therapeutics* reports results of a subsequent study in MPTP-treated monkeys in which sumanirole shows antiparkinsonian effects comparable with existing dopaminergic therapies without inducing dyskinesias using both behavioral and pathological assessments (Stephenson et al., 2005).

Conclusions

Sumanirole represents the first dopamine agonist with high selectivity for the D_2 receptor subtype over the other closely related D_3 and D_4 receptor subtypes. In vitro studies demonstrate that sumanirole is a selective D_2 receptor agonist and can therefore be used to evaluate the importance of the D_2 receptor in the physiological effects of dopamine agonists. The D_2-selective nature of sumanirole is assumed to allow for the assessment of D_2 agonist effects on locomotor activity in the absence of D_3 and D_4 receptor coactivation. Studies presented here suggest that, in animals, many of the physiological responses thought to be mediated via the D_2 receptor subtype, namely, prolactin release, regulation of striatal acetylcholine content, and autoreceptor-mediated inhibition of nigrostriatal firing rates, are indeed mediated by the molecularly defined D_2 receptor, since sumanirole was fully efficacious at in vivo concentrations expected to selectively activate D_2 receptors. Sumanirole thus represents a potential tool for studying D_2 receptor subtype specificity both in vitro and in vivo.

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